EFFECTS OF FERMENTATION TIME AND TEMPERATURE ON BUTANOL PRODUCTION FROM PALM OIL MILL EFFLUENT (POME) BY USING *CLOSTRIDIUM ACETOBUTYLICUM*

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ABSTRACT

Malaysia is the world's largest producer of palm oil. Waste from palm oil production factories has been increasing rapidly and one of the serious problems in the palm oil fruit processing is the managing of the waste generated by the processes. Recently, fermentative energy production from several sources of biomass has been carried out which is Acetone–butanol–ethanol (ABE) fermentation (Shinto *et al.*, 2008). Due to this, this study is focusing on the utilization of palm oil mill effluent (POME) for ABE fermentation by *Clostridium acetobutylicum* (NCIMB 13357). The main objective of this study is to develop conversion of POME into useful material which is butanol. Investigations were carried out on the effects of fermentation temperature within 35°C to 45°C and fermentation time within 48 to 72 hours to the butanol production. The experiment were conducted in Schott bottle with 90% concentration of culture medium and 10% v/v of inoculums concentration by using POME and reinforced clostridia medium (RCM) as growth medium. This anaerobic fermentation was done in batch culture. It was observed that the optimum condition for butanol fermentation by *C.acetobutylicum* is at 40°C through 48 hours which produced the highest yield of butanol (3.1344 g/L). From this study, it showed that the butanol production was decreased as the fermentation temperature and time were increased. The results from this study hence showed that the POME is a viable media for butanol fermentation. For improvement, research on other parameter such as pH of substrate and concentration of substrate that effect on butanol production should be carried out to optimize the production of butanol.

ABSTRAK

Malaysia merupakan pengeluar minyak sawit yang terbesar di dunia. Sisa buangan dari kilang pengeluaran minyak sawit telah meningkat dengan cepat. Salah satu masalah serius dalam pemprosesan minyak sawit adalah pengurusan sisa yang dihasilkan oleh proses itu sendiri. Kebelakangan ini, penghasilan pelarut asiton, butanol dan etanol (ABE) daripada sisa buangan kilang sawit telah dilakukan. Kajian telah dijalankan adalah untuk memanfaatkan sisa kilang kelapa sawit (POME) untuk fermentasi ABE oleh *Clostridium acetobutylicum* (NCIMB 13357). Tujuan utama kajian ini dilakukan adalah untuk menukarkan POME kepada bahan yang bermanfaat iaitu butanol. Eksperimen dilakukan pada pengaruh lingkungan suhu 35°C hingga 45°C dan lingkungan masa 48 hingga 72 jam untuk penghasilan butanol yang tertinggi. Kajian dilakukan di dalam botol Schott dengan kepekatan medium 90% dan 10% kepekatan inokulum dengan menggunakan media fermentasi efluen loji minyak sawit (POME) dan dikawal oleh medium kawalan (RCM) sebagai media pertumbuhan *Clostridium acetobutylicum* dalam ruangan anaerobik untuk mengekalkan keadaan anaerobik. Diamati bahawa keadaan optimum untuk fermentasi butanol oleh *C.acetobutylicum* ialah pada suhu 40°C selama 48 jam untuk menghasilkan butanol yang tertinggi iaitu sebanyak 3,1344g/L. Dari ujikaji ini menunjukkan bahawa pengeluaran butanol menurun apabila suhu fermentasi dan masa meningkat. Hasil dari kajian menunjukkan bahawa POME adalah media yang sesuai untuk penghasilan butanol.

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LIST OF SYMBOLS / ABBREVIATIONS

ABE	-	Acetone-Butanol-Ethanol
ATP	-	Adenosine triphosphate
adc		Acetoacetic acid decarboxylase
C.acetobutylicum	-	*Clostridium acetobutylicum*
CO_2		Carbon dioxide
DNS	-	Dinitrosalicylic Colorimetric Method
GC		Gas Chromatography
GC-FID	-	Gas Chromatography equipped with Flame Ionization Detector
NaOH	-	Sodium Hydroxide
N_2		Nitrogen gas
OD	-	Optical Density
POME	-	Palm Oil Mill Effluent
RCM	-	Reinforced Clostridium Medium
RNA	-	Ribonucleic acid
USA	-	United States of America
UV-VIS	-	Ultraviolet-Visible Spectroscopy

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CHAPTER 1

INTRODUCTION

1.1 Background of Research

The Malaysian palm oil industry has grown rapidly over the years. Malaysia has become the world's largest producer and exporter of palm oil and its products (Wu *et al.*, 2009). Malaysia is producing more than 80% of the world's crude palm oil. Palm oil is produced from palm fruit mill through steaming and squeezing process. One of the serious problems in the palm fruit processing is the managing of the wastes generated during the processes. The waste consists of a significant amount of solid waste and a wastewater called palm oil mill effluent (POME) (Miura *et al.*, 2001). If the effluent discharged untreated, it can certainly cause considerable environmental problems. POME has great potential as a substrate for acetone-butanol-ethanol (ABE) fermentation because it contains a mixture of carbohydrates including starch, hemicelluloses, sucrose and other carbohydrates that can be utilized by saccharolytic clostridia.

The production of acetone-butanol-ethanol (ABE) by solvents producing strains of Clostridium was one of the first large-scale industrial fermentation process developed (Kalil *et al.*, 2003). The fermentation of ABE was widely carried out using renewable sources. ABE producing clostridia posses two distinct characteristic phases in energy acquiring pathway, especially acidogenesis and solventogenesis. During acidogenesis, cell growth is exponential and products are acetic acid and butyric acid with ATP formation. During solventogenesis, cell growth enters the stationary phase and the above organic acids are reutilized and acetone, butanol and ethanol are produced (Tashiro *et al.,* 2004). These metabolism pathways of clostridia offer an opportunity to establish the ABE fermentation as an economically viable process (Sillers *et al.,* 2009).

Acetone, butanol and ethanol are dominant products of the acetone-butanol-ethanol (ABE) process which butanol constitutes 60-70% (w/w) of the total solvents, acetone 20-30%, and ethanol about 10%. Butanol is the most valuable of these solvent products (Jiang *et al.,* 2009). Since butanol has remarkable features such as hydrophobicity, high energy content, and ease of storage and transportation, it has been proposed as a substitute and supplement of gasoline as a transportation fuel (Shinto *et al.,* 2008). Current utilization strategies for biomass have focused on ethanol production; producing butanol instead of ethanol offers several advantages for biofuel-gasoline blending. Butanol has lower vapor pressure but higher energy content than ethanol, which makes the former safer for blending with gasoline as well as offering better fuel economy than ethanol-gasoline blends (Hipolito *et al.,* 2008).

Butanol has a higher tolerance to water contamination in gasoline blends and therefore butanol-gasoline blends are less susceptible to separation and that facilities its use in existing gasoline supply and distribution channels. Furthermore, butanol can be blended with gasoline at higher concentrations than ethanol without the need to retrofit vehicles. Therefore, optimizing acetone-butanol-ethanol (ABE) fermentation to enhance butanol production over ethanol appears to be more commercially and technologically attractive opinion. Besides that, palm oil mill effluent (POME) utilization would further increase profitability of palm oil mill industry and solve an environmental problem. The availability of cheap and readily available sources of substrate such as POME should enhance the economic viability of fermentation process for ABE production (Takriff *et al.*, 2009).

1.2 Problem Statement

This research is proposed to convert waste into wealth. Malaysia is producing more than 80% of the world's crude palm oil. One of the serious problems in the palm fruit processing is the managing of the wastes generated during the processes. By-products from palm oil mill constitute the most abundant renewable resources available in Malaysia. The abundance of oil palm empty fruit bunches has created a vital environmental issue. In the year 2004, more than 40 million tons of palm oil mill effluent (POME) was generated from 372 mills in Malaysia. If the effluent is discharged untreated, it can certainly cause considerable environmental problems due to its high biochemical oxygen demand (25,000 mg/l), chemical oxygen demand (53,630 mg/l), oil and grease (8370 mg/l), total solids (43,635 mg/l) as well as suspended solids (19,020 mg/l).

The palm oil mill industry in Malaysia is identified as the one that produces the largest pollution load into the rivers throughout the country. The discharge of untreated palm oil mill effluent (POME) though creates adverse impact to the environment, the notion of nurturing POME and its derivatives as valuable resources should not be dismissed (Wu *et al.*, 2007). Besides that, it is also an efficient solution when using POME as an alternative to produce a butanol because it is a renewable fuel instead of using fossil fuel. Depleting non-renewable natural resources such as mineral petroleum has raised the concern for the need of renewable sources to overcome the shortage of transportation fuel in the coming era and at the same time to protect the environment from pollutants (Fei *et al.*, 2008).

Butanol is an important industrial solvent and potentially a better fuel extender than ethanol. In the 1970s, the primary focus for alternative fuels was on ethanol. People were familiar with its production and did not realize that dehydration was necessary in order to blend it with fossil fuels. Nor did people realize the difficulty of distribution, since ethanol cannot be transferred through the existing pipeline infrastructure. The selection of ethanol, a lower-grade and corrosive, hard-to-purify, dangerously explosive, and very evaporative alcohol is the result. Ethanol is still subsidized by the government, since it is not profitable enough to compete with gasoline.

On the other hand, butanol which is a new alternative is the good biofuel which when it consumed in an internal combustion engine yields no carbon monoxide all environmentally harmful byproducts of combustion. Carbon dioxide (CO_2) is the combustion byproduct of butanol, and is considered environmentally 'green' (Fei *et al*., 2008). Butanol is far less corrosive than ethanol and can be shipped and distributed through existing pipelines and filling stations. Reformed butanol has four more hydrogen atoms than ethanol, resulting in a higher energy output and is used as a fuel cell fuel. Current butanol prices as a chemical are at $3.75 per gallon, with a worldwide market of 370 million gallons per year. The market demand is expected to increase dramatically if green butanol can be produced economically from low cost biomass.

Widespread adoption of butanol as an alternative fuel to replace gasoline would stimulate an increased demand for corn and other organics as well as waste biomass. In this research, palm oil mill effluent (POME) is used as an alternative to produce a butanol instead of using corn because corn will contribute a food crisis. Numerous carbon sources such as glucose, molasses, starch and corn has been utilized in the acetone-butanol-ethanol (ABE) fermentation. However the cost of substrate has a dramatic influence on the economic viability of ABE production via fermentation (Ezeji *et al.,* 2007 and Takriff *et al.,* 2009).

Many developed countries heavily subsidies the growing of food crops to produce ethanol before. However, the subsidy for corn has reduced the land used for the production of other food crops such as wheat and soya. Therefore, world prices of all these crops have gone up sharply. This, in turn, has affected millions of poor people all over the world in terms of their ability to have access to affordable food. The public too must play a role by reducing their consumption of resource-intensive food. Thus, the availability of cheap and readily available sources of substrates such as palm oil mill effluent (POME) should enhance the economic viability of fermentation processes for acetone-butanol-ethanol (ABE) production (Takriff *et al.,* 2009).

Therefore, solution should be found and developed. Various technically feasible means of transforming the palm oil mill effluent (POME) into different benefit products through cleaner production and environmentally sound biotechnologies for enabling the balance between environmental protection and a sustainable reuse of biosources found in POME (Wu *et al.,* 2009).

1.3 Objective

The main objective for this research is to study the effects of fermentation temperature and fermentation time in producing higher butanol from the anaerobic fermentation of Palm Oil Mill Effluent (POME) by using *Clostridium acetobutylicum*.

1.4 Scopes of research

The main scopes of this research are:

(i) To study the growth profile of *Clostridium acetobutylicum.*

(ii) To study the effect of fermentation time within the range 48 to 72 hours to the butanol production.

(iii) To study the effect of fermentation temperature within 35°C to 45°C to the butanol production.

(iv) To study the glucose consumption in fermentation broth by using dinitrosalicylic acid (DNS) method.

1.5 Rationale and Significance

(i) This research applies concept of ‘waste to wealth’ due to abundant supply of palm oil mill effluent (POME) as a substrate to yield butanol in huge amount.

(ii) Butanol is an alternative to replace the use of ethanol which butanol has a lot of advantages compare to ethanol.

(iii) The increased demand of corn for bioethanol fuel production will contribute a food crisis. The price of corn will rapidly increase and people will suffer for food.

CHAPTER 2

LITERATURE REVIEW

2.1 Butanol

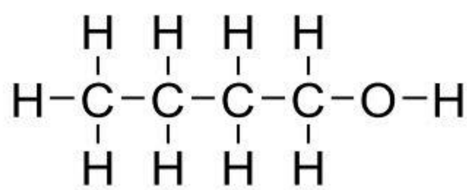

Figure 2.1 Structure of butanol

Butanol is a 4-carbon alcohol that has more energy than ethanol; 25% more energy per unit volume (Shapovalov and Ashkinazi, 2008). Butanol is a cleaner and superior extender than ethanol with octane numbers 113 and 94 as compared with that of 111 and 94 for ethanol (Qureshi *et al.*, 2008). Butanol can be produced from renewable resources such as corn, sugar beets, sorghum, cassava, sugarcane, corn stalks, and other biomass. Unlike conventional biofuels, such as ethanol and biodiesel that are mainly derive from food-based feedstocks, biobutanol is an advanced biofuel that can be derived from non-food sources.

Butanol is produced by solventogenic clostridia via the acetone-butanol-ethanol (ABE) fermentation. Historically, butanol (or ABE in fermentation broth, the typical ratio of ABE is 3:6:1, where butanol is a major product) fermentation is second to ethanol and there were plants that operated during World War I (Qureshi *et al.,* 2008). Butanol is now recognizing as an important transport fuel with superior characteristics to ethanol. Its inherent chemical properties make it superior to ethanol for use in combustion engines:

i. Butanol has a lower vapor pressure and higher flashpoint than ethanol, making it easier to store and safer to handle.
ii. Butanol is safer to handle with a Reid Value of 0.33 psi, which is a measure of a fluid's rate of evaporation when compared to gasoline at 4.5 and ethanol at 2.0 psi.
iii. Butanol is less corrosive than ethanol and can be transported using existing infrastructures.
iv. Butanol when consumed in an internal combustion engine yields no carbon monoxide, all environmentally harmful byproducts of combustion. CO_2 is the combustion byproduct of butanol, and is considered environmentally 'green' (Shapovalov and Ashkinazi, 2008).
v. Butanol is an industrial commodity, with 370 million gallons per year market with a selling price of $3.75 per gallon.
vi. Hydrogen generated during the butanol fermentation process is easily recovered, increasing the energy yield of a bushel of corn by an additional 18 percent over the energy yield of ethanol produced from the same quantity of raw material.
vii. Butanol is a pure alcohol with energy content similar to that of gasoline. It does not have to be stored in high-pressure vessels like natural gas (Hipolito *et al.*, 2008).
viii. Butanol is more “hydrophobic” than ethanol, meaning it has a higher tendency to repel water. This quality allows it to blend well with gasoline, could be used to

improve ethanol / gasoline blending, and might mean it is potentially suitable for transport in pipelines.

On the other hand, butanol is used as an ingredient in perfumes and as a solvent for the extraction of essential oils. Butanol is also used as an extractant in the manufacture of antibiotics, hormones and vitamins and as solvent for paints, coatings, natural resins, gums, synthetic resins, dyes, alkaloids, and camphor. Other miscellaneous applications of butanol are as a swelling agent in textiles, as a component of brake fluids, cleaning formulations, degreasers, and repellents and as a component of ore floatation agents, and of wood-treating systems.

2.2 Anaerobic fermentation

The anaerobic process has been developed for the efficient treatment of waste and high organic wastewater. One of the advantages of the anaerobic process is the recovery of the useful matters such as solvent and methane (Hwang *et al.,* 2004). Given the need to reduce carbon dioxide (CO_2) accumulation in the Earth's atmosphere, anaerobic processes should attract more attention than aerobic ones because much less the former generates CO_2 (Somrutai *et al.,* 1996). Anaerobic fermentation is a promising method of sustainable hydrogen production since organic matter, including waste products, can be used as a feedstock for the process. The highest yields of hydrogen have been reported with strains of clostridia in pure cultures or mixed cultures where clostridia are predominant (Alalayah *et al.,* 2009).

Acetone, butanol and ethanol production is a strictly anaerobic process due to the strictly anaerobic Clostridium. Actually the process was industrialized for production of acetone. Then, it was used to produce butanol which has varied applications. At present, both acetone and butanol are obtained by chemical synthesis, and this fermentation is rarely used *Clostridium acetobutylicum* which has an ability to utilize various substrates. The fermentation yields number of products of which acetone, butanol and ethanol are the major ones. The fermentation process uses conversion of starch to acetone by *C. acetobutylicum*. According to Somrutai *et al.* (1996), this strain is able to convert the carbohydrates in molasses to acetone and butanol.

Normally in anaerobic fermentation, the cultivations were conducted anaerobically at 30ºC without pH control and the inoculum formed 10% (v/v) of the culture volume. For a batch fermentation which exceeding a working volume of 100 ml, the headspace of the culture was purged with oxygen-free nitrogen gas for 30 minutes after inoculation. Much anaerobic fermentation does, however, require mild aeration for the initial growth phase, and sufficient agitation for mixing and maintenance of temperature.

2.3 Acetone-butanol-ethanol (ABE) fermentation

Acetone–butanol–ethanol (ABE) fermentation was industrially carried out during the first half of last century, but was subsequently unable to compete economically with the petrochemical industry (Jones and Wood, 1986). However, there has been a revival of interest in ABE fermentation, since renewable resources as such domestic and agro-industrial wastes have become possible alternative substrates for the production of chemicals and liquid fuels (Kobayashi *et al.*, 2005).

Acetone-butanol-ethanol (ABE) fermentation by *Clostridium acetobutylicum* is one of the oldest known industrial fermentations. It is one of the largest biotechnological processes ever known. The metabolic pathways of ABE-producing clostridia consist of two distinct characteristic phases namely acidogenesis and solventogenesis (Shinto *et al.*, 2008). During acidogenesis, cell growth is exponential and products are acetic acid and butyric acid with ATP formation. During solvetogenesis, cell growth enters the stationary phase and the above organic acids are reutilized and acetone, butanol and ethanol are produced (Tashiro *et al.*, 2004). These solvents produced in a ratio of 3:6:1, or 3 parts acetone, 6 parts butanol and 1 part ethanol.

2.4 *Clostridium acetobutylicum*

Solvent-producing clostridia were used extensively from the beginning of the 20^{th} century for the industrial production of acetone and butanol (Jones and Wood, 1986). This research has remained because of their potential application in biotechnology. Strains classified as *Clostridium acetobutylicum* were the first industrial cultures to be successfully isolated, patented and used for a large scale production of solvents from starched-based substrates (Keis *et al.*, 2001). *C.acetobutylicum* is a gram-positive, spore-forming, rod shaped, forming terminal or subterminal spores and strictly anaerobic. Therefore, it will not grow in the presence of oxygen. It is able to ferment various sugars to form the highest yields of acetone and butanol from a variety starchy substrate by using the ABE process (Zhao *et al.,* 2003). It normally can be found in soils, lake sediments, well water, and bovine, canine, and human feces.

The metabolic pathways of acetone-butanol-ethanol ABE-producing clostridia consist of two distinct characteristic phase; acidogenesis and solventogenesis. A figure below shows the metabolic pathways of *C.acetobutylicum* ATCC 824 by Jones and Wood (1986). In general, during acidogenesis, ABE-producing clostridia grow exponentially, and acetic and butyric acids are produced with ATP formation. Further, in the subsequent solventogenesis, cell growth attains a stationary phase; organic acids are reassimilated and acetone, butanol, and ethanol are produced. ABE fermentation includes substrate inhibition by glucose and xylose and product inhibition by butanol and these lead to low productivity and yield of solvents (Shinto *et al.*, 2008).